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The line blot: an immunoassay for monoclonal and other antibodies

Its application to the serotyping of Gram-negative bacteria

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A procedure is described for assaying antibodies based on the application of antigen to nitrocellulose as a line with an ink pen point. The method requires no expensive apparatus, is easy to perform, and requires less than 0.25 ng of antigen per assay. More than 45 antigens can be assayed simultaneously with a single antibody. Antigens can be applied as purified proteins, extracts, or sodium dodecyl sulfate solubilized extracts. The application of the line blot assay for the detection of monoclonal antibodies which recognize heat-sensitive and insensitive epitopes on the typhus rickettsia surface protein antigen is described. A new serotyping assay for Gram-negative bacteria is also described in which sodium dodecyl sulfate solubilized antigens are applied as lines with and without prior proteinase K digestion. The value of the line blot serotyping assay is demonstrated with *Proteus*, *Rickettsia*, *Rochalimaea*, and *Legionella* antigens. The line blot immunosassy is a simple, but powerful and flexible, alternative to dot and cross-dot immunoassays.

Key words: Immunochemical method; Monoclonal antibody; Lipopolysaccharide; Protein conformation; Membrane solubilization; Line blot immunoassay

Introduction

The technique of immobilization of antigens on nitrocellulose and other adsorbents has been widely employed for studying the interactions of antibodies with these antigens. In particular, dot-immunobinding assays (Hawkes et al., 1982; Towbin and Gordon, 1984) have been widely adopted because of their simplicity and versatility. In many cases, the antigen dot is applied directly as a small 1 μl spot with a microsyringe. To permit a more uniform application of antigen as well as use of dilute antigen solutions, 96 well microplate format filtration apparatus with 3 mm diameter wells were developed (Kranz and Gennis, 1982). These have been widely commercialized. To avoid cutting sheets when multiple antigen dots were incubated with a single antibody, an aluminum template apparatus with 12 channels for antibody was developed (Smith et al., 1984). More recently, this template approach was signifi-

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Abbreviations: SDS, sodium dodecyl sulfate; SPA, rickettsial serotype protein antigen; SPA-D, denatured SPA; TOT, French pressure cell disrupted total antigen; WC, whole cell antigen; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBS, 10 mM Tris-HCl, 250 mM NaCl, 0.01% merthiolate, pH 7.5; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; MAB, monoclonal antibody.

cantly extended as the cross-dot and cross-blot assays which employed M70 Altuglass templates with up to 30 2 mm lanes (Alric et al., 1986). Both antigen application and antibody incubations could be done sequentially after 90° rotation permitting up to 600 individual assays per sheet. A recent commercial apparatus (Immunetics) employs 45 lanes, thus permitting up to 2025 assays per sheet if employed in the cross-dot manner. The limitation to this template approach is that the apparatus is expensive and must be constructed to precise tolerances to avoid well to well leakage. There is also a practical limit to the minimum width of the template channels that can be manufactured and the ease of antigen or antibody introduction to the channels and its subsequent incubation and washout.

In this paper, we introduce the line blot. No template is necessary. Antigen is rapidly applied as a thin line with a pen point. Subsequent assays, as described here, employ strips cut perpendicular to the antigen line; however, the templates described above could also be employed easily for the subsequent antibody incubations. We demonstrate the versatility of the line blot by applying it to the characterization of monoclonal antibodies and in the development of a new serotyping assay for Gram-negative bacteria. The serotyping assay employs sodium dodecyl sulfate (SDS)-solubilized cells with and without proteinase K digestion. The line blot immunoassay has also been evaluated extensively by screening human sera from various rickettsial infections against multiple bacterial antigens (Raoult, D. and Dasch, G.A., manuscripts submitted for publication). The line blot immunoassay is of interest because it is simple to perform, economical of antigen, and extremely versatile in the number of antigen-antibody reactions that can be assayed simultaneously.

Materials and methods

Antigens

Rickettsia prowazekii Breinl, *R. typhi* Wilmington, and *R. canada* McKiel were grown in the yolk sacs of embryonated chicken eggs and purified from host cell contaminants by differential centrifugation and isopycnic banding in Renografin density gradients (Dasch and Weiss, 1977; Woodman et al., 1977). Rickettsial serotype protein antigens (SPA) were released by osmotic shock of purified rickettsiae in deionized water and removal of the extracted cells by centrifugation at $17\,500 \times g$ for 15 min; the SPA was purified by ultracentrifugation at $200\,000 \times g$ for 2 h and the supernatant sterilized by passage through a 0.45 μm filter (Dasch, 1981). French pressure cell disrupted extracts of rickettsiae (TOT) were those described previously for use in ELISA (Halle and Dasch, 1980). Both TOT and SPA antigens were preserved with 0.2% formalin and stored at 4°C. Proteus OX19 (ATCC 6898), OX2 (ATCC 7829), and OXK (ATCC 15146) were grown in Nutrient Broth. *Rochalimaea quintana* was grown in suspension culture as described (Weiss and Dasch, 1982). The source, passage histories, and growth of *Legionella* strains on a buffered diphasic charcoal yeast extract agar overlaid with liquid yeast extract medium was as described (Westfall et al., 1986). Well-washed bacterial suspensions were suspended in distilled water at 500–1000 μg protein/ml and stored in small aliquots at −40°C as whole cell antigens (WC).

Antisera

Rabbit antisera used in the study were those previously described (Dasch and Weiss, 1977; Weiss and Dasch, 1982; Westfall et al., 1986) except for three commercial sera which were those provided as controls in Weil-Felix agglutination kits for antibodies to Proteus OX19, OX2, and OXK (Difco Laboratories). Hybridomas were produced by conventional HAT selection techniques by polyethylene glycol fusion of SP2/0-Ag14 myeloma cells with spleen cells from BALB/c or NMRI mice hyperimmunized with purified rickettsiae. Monoclonal antibodies were used as undiluted culture supernatants of limiting dilution cloned hybridomas grown in RPMI 1640 with 10% fetal bovine serum. Monoclonal antibodies were stored at 4°C with 0.02% thimersol as preservative. Details of their selection and properties (Table I) will be described in detail elsewhere (Dasch et al., manuscript in preparation).

Antigen solubilization

WC antigens were solubilized with one half volume of Laemmli PAGE solubilizer (4% SDS, 10% 2-mercaptoethanol, 0.05% bromphenol blue,

TABLE I

CHARACTERISTICS OF TYPHUS MONOCLONAL ANTIBODIES USED IN LINE BLOT IMMUNOASSAY

Antibody	Typhus specificity [a]	Immunoglobulin subclass	Western blot		Heat sensitive epitope [d]
			Antigen [b]	Specificity [c]	
a: T11-3C7.1	T only	KG2a	SPA	T only	Yes
b: T66-1E8.1	T only	KG1	SPA	T only	No
c: T1-6A5.1	T > P	KG1	SPA	T = P	Partial
d: P46-5D10.1	T = P	KG1	SPA	P > T	No
e: P46-3A2.1	P > T (mixed)	KG1	SPA	P only	Yes
			LPS	T = P	No
f: P51-4B12.1	C > T = P	KG1	SPA	C = T = P	No
g: C67-5F5.1	C only	KG1	SPA	C only	No
h: T66-1D12.1	C = T = P	KG3	SPA	C = T = P	No
i: P46-3F5.1	T = P	KG3	LPS	T = P	No
j: P46-2B7.1	T = P > C	KG2a	LPS	T = P > C	No
k: T5-4B11.3	T = P = C	KG2a	60K	T = P = C	No
l: C9-1E7.1	C only weak	KG3	SPA	Weak C only	Yes
m: C9-4A9.1	T = P = C	KG3	LPS	T = P = C	No
n: T65-1G2.2	T only	KG1	SPA	Not done	No
o: T66-1C10.1	T only	KG2a	SPA	T > P = C	No
p: TP29-6C3.2	T = P	KG1	SPA	T = P	Partial
q: T66-2D3.1	T = P	KG2b	SPA	T = P	No
r: P52-3H5.2	P only	KG1	SPA	P only	Partial
s: P53-2G12.2	P only	KG1	SPA	P ≫ T	No
t: C68-4F6.1	Lost reactivity	–	–	–	–
u: P50-2B8.1	T = P	KG1	SPA	T = P	Partial
v: P46-4D1.1	T = P	KG3	LPS	T = P	No
w: P46-3C10.3	T = P = C	KG3	LPS	T = P > C	No
x: P46-3F11.2	T = P = C	KG1	LPS	T = P > C	No
y: Medium control	–	–	–	–	–

[a] ELISA reactivity at 1/12 dilution. T = *R. typhi*, P = *R. prowazekii*, C = *R. canada*.

[b] SPA = species-specific protein antigen; 60K = 60 kDA common bacterial protein antigen; LPS = lipopolysaccharide.

[c] Species identified as in [a] but evaluated without dilution.

[d] Change in Western blot reactivity of samples solubilized by boiling prior to PAGE relative to those solubilized at room temperature.

0.125 M Tris HCl pH 6.8, 25% glycerol) and one half volume of Tris-buffered saline (10 mM Tris-HCl, 250 mM NaCl, 0.01% merthiolate) (TBS) with or without boiling for 5 min.

Proteinase K treatment of antigens

WC antigens (1 mg protein/ml of water) were boiled with $\frac{1}{4}$ vol. of Laemmli PAGE solubilizer and then incubated at 60 °C with $\frac{1}{4}$ vol. of proteinase K (2.5 mg/ml in TBS) (Boehringer Mannheim Biochemicals) for 1 h followed by a second identical addition and 60 °C incubation with proteinase K. Finally the digested sample was boiled again for 10 min.

Application of antigens to nitrocellulose

BA85 nitrocellulose (0.45 μm) (Schleicher and Schuell, Keene, New Hampshire) sheets were cut to a rectangular size appropriate for the experiment. TOT and SPA antigens were dispensed directly in wells of 96 well microtiter plates. Since WC antigens were still potentially infectious, they were always used as SDS solubilized antigens with or without proteinase K digestion or boiling. However, in principle, there is no difficulty in using the irradiated, formalin-treated or autoclaved whole cell antigens utilized in dot-blot or ELISA assays so long as they are a homogeneous suspension permitting uniform application. Anti-

gen application to the nitrocellulose was most easily effected with a pen holder and removeable tips. Pen tips causing the least effacing of the nitrocellulose were those with a round extremity (Speedball pens, round gothic letter style, B6, Hunt manufacturing Co., Statesville, NC 28677). Pen tips were washed in distilled water and dried before use; they can be reused indefinitely with proper cleaning. Antigen was drawn from the well with the pen tip and applied as a line using a ruler. Lines with different antigens were generally spaced about 0.3–0.5 cm apart. Using a commercially available incubation tray with 25 milled wells (10 × 228 × 22 mm) (Bio-Rad Laboratories, Richmond, CA; no. 170-4037), 45 antigen lines were easily assayed on 140 × 4 mm strips cut perpendicular to the antigen lines. The amount of protein deposited was easily monitored by (a) India ink staining (0.1% Pelikan India ink in 0.5% Tween 20 in PBS) (Hancock and Tsang, 1983); (b) reversible Ponceau S staining (1.0% dye in 1% acetic acid in water) (Salinovich and Montelaro, 1986); or (c) avidin-biotin protein assay (Bio-Rad Laboratories, Richmond, CA, no. 170-6512) or other hapten derivatization methods (Wojtkowiak et al., 1983). The reversible Ponceau S stain had the advantage that a sheet could be stained and the dye removed during the first step of the immunodetection procedure (milk blocking).

Line blot immunoassay

Nitrocellulose sheets with antigen lines were blocked to prevent non-specific antibody binding with 5% non-fat dry milk in TBS for 1 h. Sheets were then dried and stored for later use or cut directly with a paper cutter into strips of the desired width. Each strip was incubated with 2 ml of antibody (150 × 10 mm well incubation tray) or 4 ml of antibody (228 × 10 mm well incubation tray) and incubated 2 h to overnight (polyclonal sera 1/500 in 3% milk in TBS; monoclonal culture supernatants undiluted or up to 1/10 in TBS) at 30 °C. After removal of the antibody, strips were washed three times in TBS for 10 min each. Anti-immunoglobulin horseradish peroxidase conjugates (Bio-Rad Laboratories, Richmond, CA or Sigma Chemical Co.) were diluted 1/500–1/3000 in 3% milk in TBS and incubated with the strips for 2 h at 30 °C. After three 10 min washes in TBS the strips were incubated in 0.015% 4-chloro-1-naphthol and 0.015% hydrogen peroxide in 16.7% methanol in TBS for 10–15 min (Hawkes et al., 1982). Finally, the strips were thoroughly rinsed in water, dried between filter paper and photographed. Stained sheets were dried quickly and stored in the dark to avoid development of yellow background and loss of blue specific stain on the nitrocellulose.

Results

Quantity of antigen required in assay

To determine the amount of antigen required for simple visualization of antibody binding, antigens were serially diluted, applied as regularly spaced lines, and immunodetected with monoclonal and polyclonal antisera followed by horseradish peroxidase anti-immunoglobulin conjugates and histochemical enzyme staining with 4-chloro-1-naphthol (Fig. 1). Using TOT and SPA rickettsial antigens, positive test sera gave strong signals with antigen concentrations of 125–500 μg protein/ml (Fig. 1, lines 2–4; Fig. 2) although considerably less antigen (Fig. 1, lines 5–9) gave signals distinguishable from background with some sera. With minimal practice, lines exhibiting little variation in width except at the very beginning and end of the lines could be obtained. These termini were generally trimmed from the test samples. With a smooth motion using a straight edge as guide, a line of 41 cm could be obtained from 50 μl of antigen. Consequently, a single test of 4 mm of line required about 0.5 μl of a 500 μl/ml antigen solution. This is about the same amount (0.25 μg) employed in a single microtiter well for an ELISA assay with these antigens. SDS solubilized antigens as well as crude lipopolysaccharide (LPS) preparations obtained by proteinase K digestion could be used at comparable antigen loadings (Fig. 3). Although TOT and SPA antigens dried instantly on the paper, the SDS and glycerol in the Laemmli solubilizer caused the paper to dry less quickly and the liquid to form an apparently wider line on the nitrocellulose. However, the protein (as monitored most conveniently with Ponceau S) bound quite rapidly and formed a line scarcely wider than that obtained with antigen applied in

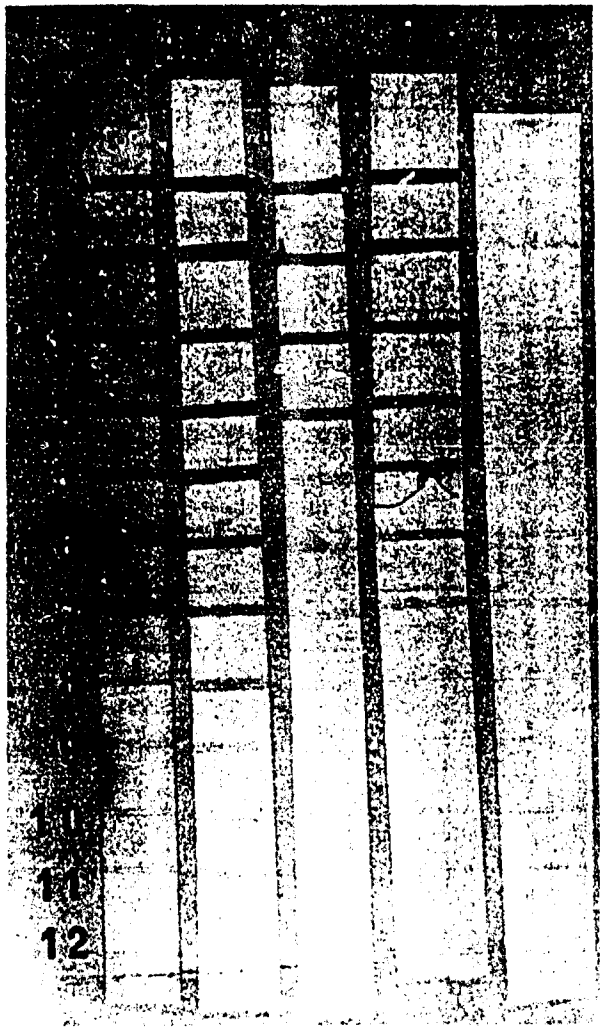

Fig. 1. Antigen requirements of line blot assay. *R. typhi* TOT antigen was diluted two-fold serially in phosphate-buffered saline from 1000 μg/ml (line 1) to 0.5 μg/ml (line 12). A: India ink stained strip. B–E: milk blocked strips immunodetected with specific antisera, conjugates, and 4-chloro-1-naphthol stain. B: convalescent typhus serum from human case. C: monoclonal antibody T11-3C7.1 against heat-sensitive *R. typhi*-specific SPA epitope. D: monoclonal antibody T66-1E8.1 against heat-insensitive *R. typhi*-specific SPA epitope. E: negative human control serum.

the absence of SDS (compare Figs. 1 and 2 with Fig. 3). The described technique of solubilization and proteinase K digestion was chosen since these materials are available in most laboratories conducting PAGE and Western blotting on bacterial antigens and can be conveniently conducted on the same or parallel samples prior to PAGE analysis.

Screening of monoclonal antibodies

The tremendous versatility of the line blot immunoassay in the types and number of antigens that can be applied to a single assay strip is demonstrated in Fig. 2. TOT, SPA, and heat-denatured SPA (SPA-D) antigens of *R. typhi*, *R. prowazekii*, and *R. canada* were reacted with monoclonal antibodies selected following their characterization by ELISA and Western blotting (Table I; G.A. Dasch et al., manuscript in preparation). Because the pen leaves a faint impression on the nitrocellulose it is very simple to reassemble the original paper and to compare different antigen reactivity patterns. Remarkably little variation was observed in assays with duplicate antigen lines, even when only weak reactions were observed (Fig. 2). MAB reactions by line blot generally resembled those anticipated from ELISA and Western blotting (Table I) but minor variations were detected. By line blot only two of the 24 MAB (Fig. 2l, t) failed to react appreciably more than the control medium lane (Fig. 2y): MAB l had moderate reactivity with a heat-labile epitope on SPA while MAB t was no longer making antibody after it was recultured from frozen cells. Species-specific MAB which reacted with heat-labile (*R. typhi*., Fig. 2a) or heat-insensitive SPA epitopes (*R. typhi*, Fig. 2b, n; *R. canada*, Fig. 2g; *R. prowazekii*, Fig. 2r, s) were easily recognized. Many other MAB had varying affinities for epitopes which are present on one, two or all of the three typhus group rickettsial species (compare LPS reactions among Fig. 2i and j, m and v through y; SPA reactions among Fig. 2b, e and f; 60 kDa common antigen Fig. 2k). In some cases the relative binding among the three typhus species appeared to resemble the ELISA specificity data more closely than obtained by Western blotting (Table I, Fig. 2c, f, w, x); with other MABs, the opposite pattern was true (Table I, Fig. 2d, e, o, s). In addition to providing information on the specificities of the MABs, the wide range of intensities of MAB line blot reactions made rapid selection of antibodies with unusually strong reactions or asymmetric affinities very easy (Fig. 2c, g, h, n, s, u).

Bacterial serotyping assay

Many species of Gram-negative bacteria pro-

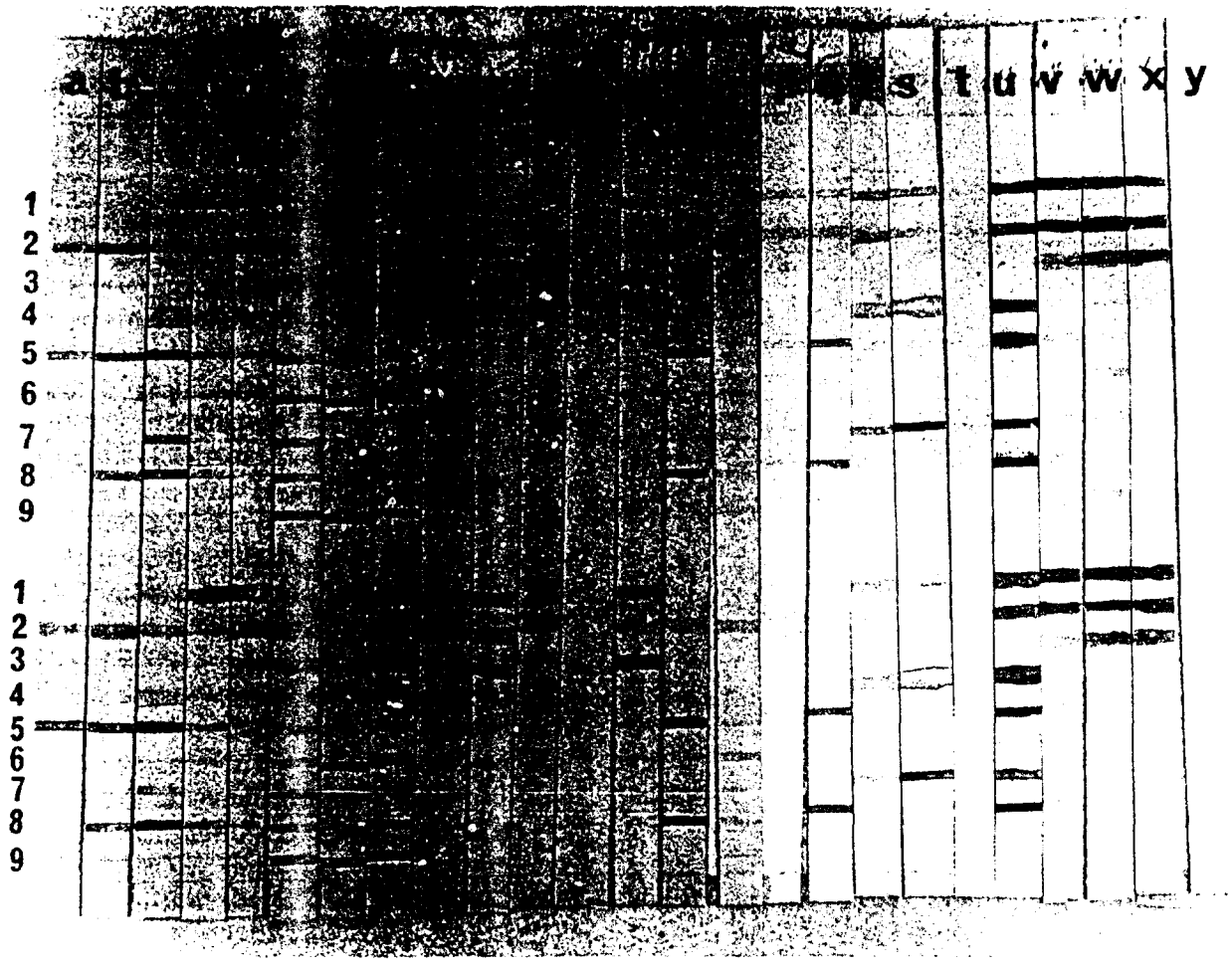

Fig. 2. Line blot immunoassay of monoclonal antibodies. Antigens diluted to 500 μg protein/ml in PBS were applied as duplicate lines and reacted with 23 different monoclonal antibodies (a–x, Table I) or control tissue culture fluid (y). *R. prowazekii* antigens: TOT (1), SPA (4), and D-SPA (7). *R. typhi* antigens: TOT (2), SPA (5), and D-SPA (8). *R. canada* antigens: TOT (3), SPA (6), and D-SPA (9).

duce diverse chemical and serological types of lipopolysaccharide (LPS). Proteinase K digestion of solubilized bacteria is a simple method for obtaining small amounts of LPS for molecular analysis by SDS-polyacrylamide gel electrophoresis and Western blotting (Hitchcock and Brown, 1983; Driver and Lambert, 1984). The line blot reactivity of rabbit sera to SDS-treated whole cell antigens from a series of *Proteus, Rochalimaea, Rickettsia*, and *Legionella* strains (regions 1 and 2, Fig. 3) was compared to that of proteinase K digested whole cells (region 3, Fig. 3). Negative control sera did not exhibit significant reactivity to any of the antigens (sera 1 and 17, Fig. 3). As had been observed previously with untreated antigens (Fig. 2), even weak reactions with SDS-treated whole cells exhibited excellent reproducibility (Fig. 3, regions 1 and 2). With the exception of the rickettsiae (Fig. 3d–f), the dominant line reaction of whole cells or proteinase K-released LPS fell on a diagonal indicating that the homologous antigen reactions were stronger than the reactions to heterologous antigens. This is in agreement with the view that LPS is the serotyping antigen of *Proteus* and *Legionella*. Proteinase K treatment greatly reduced the weaker whole cell cross-reactivities of these strains (compare region 3 with 1 and 2, Fig. 3), a finding again consistent with the known cross-reactivity of protein antigens within these two genera (Driver and Lambert, 1984; Ciesielski

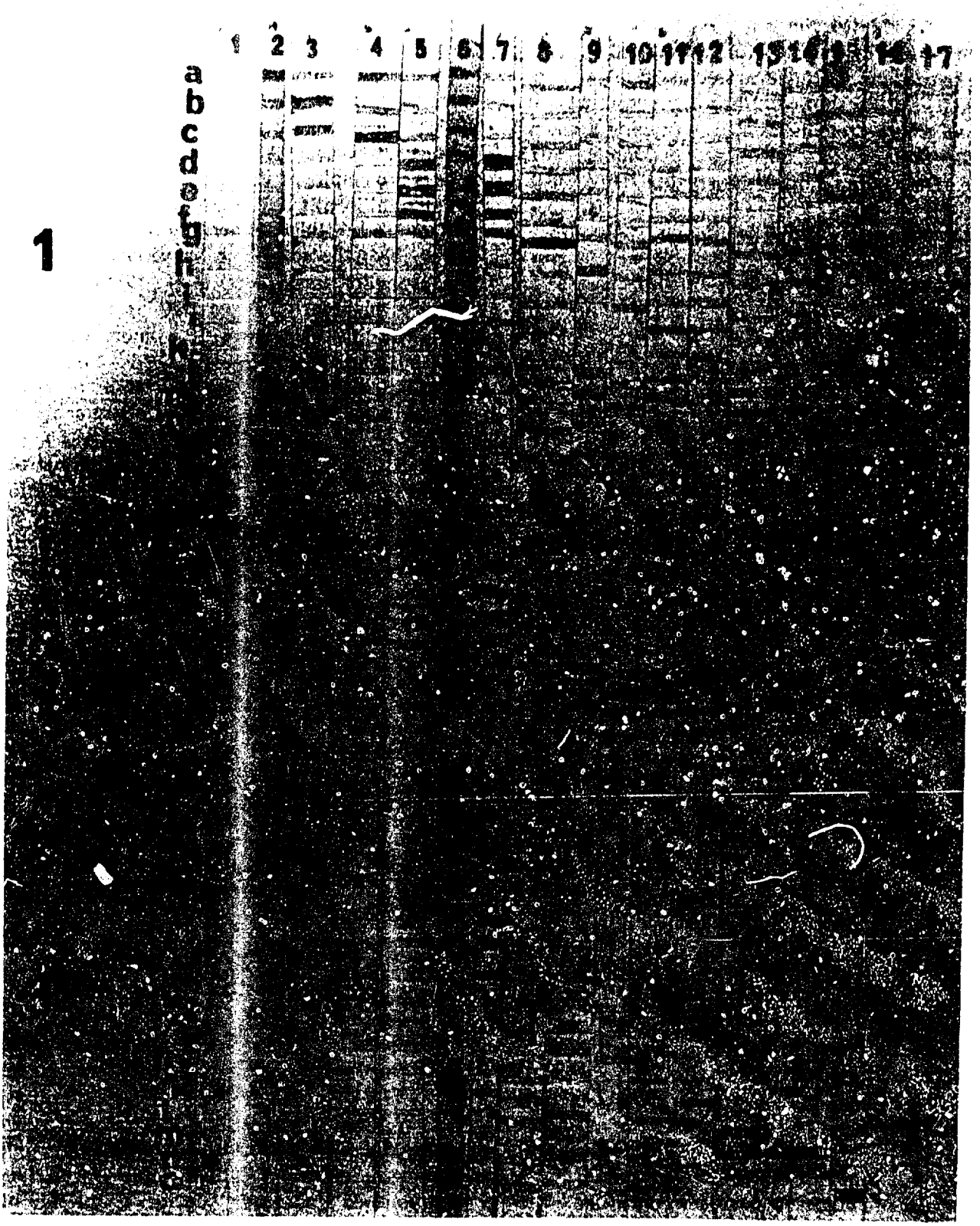

Fig. 3. Bacterial line blot serotyping assay with rabbit antisera. Boiled, SDS-solubilized WC antigen (500 μg/ml) was applied to nitrocellulose as lines without (a–e, regions 1 and 2) and with proteinase K digestion (a–e, region 3). Rabbit antisera and line antigens are respectively: *Proteus* OX2 (2, a), *Proteus* OX 19 (3, b), *Proteus* OXK (4, c), *Rickettsia typhi* (5, d), *R. canada* (6, e), *R. prowazekii* (7, f), *Rochalimaea quintana* (8, g), *Legionella bozemanii* WIGA (9, h), *L. pneumophila* strains Chicago (10, i), Dallas (11, j), Los Angeles (12, k), Philadelphia (13, l), Bloomington (14, m), Togus (15, m), and *L. micdadei* Tatlock (16, o). Sera 1 and 17 are non-immune control sera (1, rabbit; 17, goat).

et al., 1986). On the other hand, proteinase K severely diminished the strong whole cell signal of *Rickettsia* and *Rochalimaea* (Fig. 3d–g) and the rickettsial LPS showed strong cross-reactivity. In these genera proteins are the serotyping antigens while the LPS is group reactive (Dasch, 1981; G.A. Dasch, unpublished observations).

Discussion

Because the visibility of a positive dot immunoassay spot depends on the contrast of the color developed relative to the background, the size of the spot is unimportant compared to the concentration of the antigen applied (Towbin and Gordon, 1984). However, repetitive application of dots as very small volumes is tiresome and can result in variable spot shapes and ring artifacts. Further, the subsequent use of 96 well or channel templates requires precise control of the location of the small spots. Although the controlled application of antigen by means of templates with narrow width channels (Alric et al., 1986) conserves antigen relative to large 96 well filtration templates, half of the applied antigen is not available for reaction in the cross-dot format (Alric et al., 1986). The line blot nitrocellulose assay offers several advantages over dot immunoassays in time, economy, and geometry. Many laboratories already have experience with dip pen point application of small spots of particulate antigens to glass slides for use in microimmunofluorescent antibody procedures (Philip et al., 1978). Using the wet line evident with water on nitrocellulose as a guide, only a few minutes of practice are required to routinely apply lines of antigen with uniform thickness. The pen trace leaves a faint negative relief on the nitrocellulose which makes uniform spacing of parallel lines quite simple. A very large number of identical sheets with numerous antigens can be prepared in a few hours and stored for later use. Because the antigen line has very little thickness relative to antigens deposited by template, it is similar to microliter drop assays in the economy of antigen. When lanes are cut for subsequent assays, there is no loss of antigen available for reaction in contrast to the cross-dot template system. Non-specific reactions can still be detected easily because of the large amount of inter-line space. Variations in line antigen spacing can be used for internal orientation or intermediate lines drawn in ink or pencil can be used to separate groups of antigen lines. Although some antigen is wasted when antibody channel templates are employed in the antibody reaction step, it is significantly less than in cross-dot assays. When used in this manner, the line blot incorporates all of the advantages in geometry found in the cross-dot assay (Alric et al., 1986). However, since a manufactured template is not required with the line blot assay, it is more flexible in meeting varied experimental needs. Finally, the geometry of the line blot lends itself to any of wide spectrum of densitometry instruments and even, perhaps, barcode readers.

The value of the line blot is also considerably enhanced by the variety of antigen preparations which can be employed. The line blot easily distinguished antibodies recognizing heat-sensitive and insensitive epitopes present on rickettsial SPAs and would be suitable for titrating the temperature of lability of the epitope by use of lines of antigen heated to different temperatures, much as has been done in a dot assay for *Rickettsia rickettsii* SPA epitopes (Anacker et al., 1987). SDS has been used previously to disrupt *E. coli* cells so that cytoplasmic proteins and non-exported cloned proteins produced by recombinant DNA technology could be detected by dot assay (Kranz and Gennis, 1982). We have found that SDS solubilization with or without proteinase K digestion of proteins is a very simple method for preparing bacterial antigens suitable for serotyping purposes. The amount of antigen required is substantially less than classical bacterial agglutination procedures and the assay is simpler to perform than microimmunofluorescent antibody procedures, particularly when a strain is to be compared with ten or more reference strains. The line blot assay has been very useful in detecting antigenic cross-reactions among distantly related groups of bacteria and in determining their protein or polysaccharide nature (Raoult, D., and Dasch, G.A., unpublished studies). In conclusion, the line blot immunoassay is a simple, but powerful and flexible, alternative to dot and cross-dot immunoassays.

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